

Plasma Folate Concentrations Are Positively Associated with Risk of Estrogen Receptor β Negative Breast Cancer in a Swedish Nested Case Control Study^{1,2}

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Abstract

Folate's role in breast cancer development is controversial. Not only estrogen receptor (ER) α status, but also ER β status of tumors may have confounded results from previous epidemiological studies. We aimed to examine associations between plasma folate concentration and postmenopausal breast cancer defined by ER status. This nested case-control study, within the Malmö diet and cancer cohort, included 204 incident breast cancer cases with information on ER α and ER β status determined by immunochemistry on tissue micro-array sections. Plasma folate concentration was analyzed for the cases and 408 controls (matched on age and blood sample date). Odds ratios (OR) for ER-defined breast cancers in tertiles of plasma folate concentration were calculated with unconditional logistic regression. All tests were 2-sided. Women in the third tertile of plasma folate concentration (>12 nmol/L) had higher incidence of ER β - breast cancer than women in the first tertile (OR: 2.67; 95% CI: 1.44–4.92; *P*-trend = 0.001). We did not observe significant associations between plasma folate concentration and other breast cancer subgroups defined by ER status. We observed a difference between risks for ER β + and ER β - cancer (*P*-heterogeneity = 0.003). Our findings, which indicate a positive association between plasma folate and ER β - breast cancer, highlight the importance of taking ER β status into consideration in studies of folate and breast cancer. The study contributes knowledge concerning folate's multifaceted role in cancer development. If replicated in other populations, the observations may have implications for public health, particularly regarding folic acid fortification. *J. Nutr.* 140: 1661–1668, 2010.

Introduction

The B-vitamin folate has a complex role in carcinogenesis (1) and the impact of differences in folate status on breast cancer development is still largely unknown.

Plasma folate mainly occurs as 5-methyl tetrahydrofolate (5-methyl THF).⁸ This folate metabolite may be of importance in

cancer development, because it transmits methyl groups for DNA methylation and could thereby affect the expression of different genes involved in the cancer process, such as tumor suppressor genes (2). In addition, plasma folate is a marker of total folate status (3) and therefore also reflects the concentration of another folate metabolite, 5,10-methylene tetrahydrofolate. This form could be important in cancer development via its involvement in DNA synthesis and repair (4).

Folate intake (5), as well as genetic variation of the folate metabolizing enzyme methylene tetrahydrofolate reductase (MTHFR) (6), influence plasma folate concentrations. MTHFR catalyzes the conversion of 5,10-methylene tetrahydrofolate into 5-methyl THF. The minor T allele of the *MTHFR* 677C > T (rs1801133) single nucleotide polymorphism (SNP) has been associated with reduced MTHFR activity and thereby reduced plasma folate concentrations (6,7). The minor C allele of the *MTHFR* 1298 A > C (rs1801131) has been associated with a less pronounced reduction of the MTHFR activity (8). Linkage between the 2 *MTHFR* polymorphisms has been observed (9–11).

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⁸ Abbreviations used: ER, estrogen receptor; 5-methyl THF, 5-methyl tetrahydrofolate; MTHFR, methylene tetrahydrofolate reductase; MDC, Malmö diet and cancer; MHT, menopausal hormone therapy; OR, odds ratio; PR, progesterone receptor; SNP, single nucleotide polymorphism.

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Breast cancer studies on plasma folate, folate intake, and the *MTHFR* 677C > T polymorphism among postmenopausal women are conflicting (12,13). Some epidemiological studies suggest that high intakes are protective (12). Meta-analyses do not support an overall protective association but indicate protective associations among women who consume alcohol (12,13). In addition, high folate intake has been associated with increased breast cancer risk in a large prospective study (14).

Few studies have considered the fact that there are different types of breast tumors, which may be caused by different genetic and environmental factors. An example of differences in breast cancer characteristics is the expression of sex hormone receptors.

Folate may influence methylation of estrogen receptor (ER) genes and thereby affect silencing of these genes (15). Folate-breast cancer associations may therefore differ according to ER receptor status of tumors. A few studies have examined the association between folate and ER α -defined breast cancer with inconclusive results (16–20). None of them took the expression of the recently identified ER β into consideration (21).

The main purpose of this study, on postmenopausal women from the Malmö Diet and Cancer cohort, was to examine the associations between plasma folate concentrations and risk of ER α - and ER β -defined breast cancers. We also examined if the associations were influenced by the *MTHFR* 677C > T polymorphism.

Materials and Methods

The Malmö Diet and Cancer study. The Malmö Diet and Cancer (MDC) is a prospective cohort study in Malmö, a city in the south of Sweden. Women and men born 1923–1950 were invited to participate (74,138 persons). Details of the recruitment procedures and the cohort are described elsewhere (22). The participants filled out questionnaires covering socioeconomic, lifestyle, and dietary factors, registered meals and nutrient supplements during a week, and underwent a diet history interview (23,24). Nurses drew blood samples and made anthropometric measurements. During the screening period (March 1991–October 1996), 28,098 participants completed all baseline examinations (17,035 women). Informed consent was obtained from the participants. The MDC study was approved by the Ethical Committee at Lund University (LU 51–90).

Study population. Women with prevalent cancers at baseline, except those with cervix cancer in situ, were excluded. The study includes women above 55 y of age at baseline. An exact date of menopause was not available because of missing data on reported cessation of menses, imprecise cessation of menses due to menopausal hormone therapy (MHT), or lack of detailed information on hysterectomy. We chose 55 y as the definition for menopause to minimize inclusion of perimenopausal women. A total of 204 were diagnosed with invasive breast cancer during follow-up (until 31 December 2004) and had information about ER α status and ER β status of tumors. Two controls (alive, living in Sweden, and without breast cancer at the time of diagnosis of the corresponding case) were matched on age at baseline \pm 3 mo and date of blood sampling \pm 1 mo. The study was performed after approval by the Regional Ethical Review Board in Lund (567/2005).

Breast cancer case definition and confirmation. The Swedish Cancer Registry and the Southern Swedish Regional Tumor Registry provided data on case definition and ascertainment until end of follow-up. Invasive cancer was defined as all cancer except in situ cancer. Information on vital status was obtained from the National Tax Board.

Plasma folate analysis. Blood samples were drawn from nonfasting subjects at baseline in Na-heparin tubes. The plasma fraction was separated within 1 h and stored at -80°C . The plasma folate

concentration was analyzed by a 2-step immunoassay with alkaline phosphatase, enzyme marking, and magnetic separation. Details were described in a previous publication (7). Plasma folate concentrations were determined for 203 cases (99.5%) and 408 controls (100%).

DNA analysis. DNA was extracted using QiaAmp mini-kits (Qiagen) from granulocyte or buffy coat cell suspensions, derived from EDTA blood samples drawn at baseline, and subsequently stored at -80°C (25). Genotyping of the *MTHFR* SNP 677C > T and 1298A > C (rs1801131) was performed at the Clinical Chemistry Laboratory, University Hospital in Malmö on a matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) (SEQUENOM Mass-Array) using iPLEX reagents and 10 ng DNA template. All procedures were performed according to SEQUENOM standard protocols. Duplicate MALDI-TOF MS analysis was repeated on 4% of the samples with no discrepancies between repeated analyses.

In the 2% of samples unsuccessfully genotyped on the MALDI-TOF MS, genotyping was performed on an ABI PRISM 7900HT Sequence detection system (Applied Biosystems) using SNP genotyping assays on demand (26).

Genotypes were successfully determined for 99.7% of the cases and 98.1% of the controls. Genotype distributions among controls from the MDC study did not deviate from Hardy-Weinberg equilibrium (677C > T; $P = 0.71$) (1298A > C; $P = 0.40$) (26). The minor allele frequency was 30% for 677C > T and 32% for 1298A > C (26). The minor 677T allele was linked to the major 1298A allele (26).

Sex hormone receptor status analysis. ER and progesterone receptor (PR) status analysis was performed at the Centre for Molecular Pathology in Malmö. For the construction of tissue micro arrays, 2 0.6-mm tissue cores were collected from each tumor block and arranged in a recipient block using a manual tissue arrayer (Beecher). Slides were then automatically stained using ER α (prediluted anti-ER 6F11, Ventana), ER β (1:25 EMR02, Novocastra), and PR (prediluted anti-PR clone 16, Ventana) antibodies (27). Tumors were grouped into categories according to expression of ER α , ER β , and PR (0–1, 2–10, 11–50, and 51–100% positive nuclei). Receptor status of all breast tumors was evaluated in a standardized way by 1 person, thereby eliminating inter-observer variation. All arrays were evaluated independently twice and in case of discrepancy, a third examination was performed followed by a final decision, thereby reducing the potential intra-observer bias. The tumors were classified as positive (+) and negative (–) using the clinically established cutoff value of 10% positive nuclei. ER α and ER β were determined for 204 cases (65%). For the remaining cases, adequate tumor samples were not available, either because of surgery performed at other hospitals or insufficient amount of tumor material available for histopathological evaluation. These cases were categorized as unknown. PR status was known for 98% of the ER α - and β -defined cases.

Variables. This study examined plasma folate concentrations in categories defined according to tertiles of plasma folate concentrations among the controls (2.0–8.1, 8.2–11.8, and 11.9–47.2 nmol/L). *MTHFR* 677C > T and 1298A > C categories were defined as carriage of the minor allele: yes/no (e.g. 677CC or 677CT+TT).

Self-reported food and supplement intakes were converted to nutrient intakes using PC-KOST2–93 from the National Food Administration in Uppsala, Sweden, and the MDC supplement database (28). The Pearson correlation coefficients between the reference method and the MDC dietary assessment method were between 0.5 and 0.6 for most nutrients (29). Dietary folate equivalents were calculated based on the assumption that the bioavailability of synthetic folic acid consumed in a meal is 1.7 times the bioavailability of food folate (30), i.e. dietary folate equivalents = μg food folate + $(1.7 \times \mu\text{g}$ folic acid from supplement).

Information on age was obtained from the personal identification number. Age was divided into categories, with cut points at quarters of a year. Date of blood sample referred to the screening week in the MDC study. The smoking status of the participants was defined as current smokers (including irregular smokers), ex-smokers, and never-smokers. Information on total alcohol consumption was converted into a 4-category variable. Women reporting zero consumption of alcohol in a

7-d food record and indicating no consumption of any type of alcohol during the previous year were categorized as zero-reporters. The other category ranges were <15 g/d of alcohol (low), 15–30 g/d of alcohol (medium), and >30 g/d of alcohol (high). Leisure-time physical activity was assessed using a questionnaire and categorized as low, medium, and high. Household activities were estimated in h/wk and categorized (0–9, 10–19, 20–29, ≥30). Classification of socioeconomic index was collapsed into 5 categories: manual employees, nonmanual employees (low, medium, and high), and self-employed. Retired and unemployed persons were classified according to their previous positions.

BMI (kg/m²) was calculated from direct measurement of weight and height. Age at menopause was divided into 4 categories (<45, 45–50, 50–55, >55, unknown). Current MHT (yes/no) was based on the questionnaire item “Which medications do you use on a regular basis?” in combination with information on drug use from the 7-d menu book (31). Parity was the number of children, with no children in the lowest category and ≥4 in the highest. Missing values for the variables were treated as separate categories.

Statistical analysis. The SPSS statistical computer package (version 14.0; SPSS) and Stata (version 10; StataCorp) were used for statistical analyses. Plasma folate was log transformed (e-log) to normalize the distribution before analysis. Differences in baseline characteristics between ER α - and ER β -defined cases and the entire set of 408 controls were examined with ANOVA for continuous variables. Adjustments were made for age and date of blood sample. χ^2 analysis was performed for categorical variables. The distribution of women consuming folic acid containing supplements in tertiles of plasma folate concentration was examined with χ^2 analysis. Odds ratios (OR) for ER α -, ER β -, and PR-defined breast cancer in tertiles of plasma folate were computed with unconditional multinomial logistic regression, controlling for matching variables. A second model included adjustments for established risk factors and potential confounders (i.e. weight, height, household work, smoking, alcohol intake, socioeconomic status, age at menopause, parity, and MHT). The selected covariates had to be associated with invasive breast cancer in this case control study, modify the risk estimate for invasive breast cancer according to plasma folate concentration by >10%, or by prior knowledge be considered important risk factors. A

third model also included adjustments for intakes of vitamin B-12. We also made a stratified analysis according to time below or above the median time from blood sampling to diagnosis. Finally, sensitivity analyses were made excluding women with high alcohol intakes (>15g/d), women consuming folic acid containing supplements, and cases diagnosed within 1 y after the blood sampling. Wald's test was used to examine heterogeneity between the folate-breast cancer associations according to ER β subgroup. We used unconditional logistic regression to calculate OR for the joint effects of plasma folate and *MTHFR* genotypes (677C > T and 1298A > C). A test for interaction with regard to ER β breast cancer was performed between tertiles of plasma folate (treated as a continuous variable) and carriage of the *MTHFR* 677T allele. All statistical tests were 2-sided.

Results

In examination of baseline characteristics, current use of MHT was more frequent among the breast cancer cases than among the controls regardless of ER status (Table 1). Women with ER β + breast cancer were taller and spent less time on household work, and a higher percentage had a high socioeconomic status compared with the controls. In women with ER β - breast cancer, plasma folate concentrations at baseline, age at menopause, and the percentage with high alcohol intake (>30 g/d) were higher compared with the controls. The time from blood sampling to diagnosis was shorter for ER β - cases than for ER β + cases. Consumption of folic acid containing supplements was more frequent among women in the highest tertile of plasma folate concentration, 32% compared with 6 and 8% in the lower tertiles ($P < 0.001$). The mean plasma folate concentration was 12.4 nmol/L (10.8 nmol/L among women who did not use folic acid-containing supplements and 21.1 nmol/L among supplement users).

Plasma folate concentration was positively associated with risk of ER β - breast cancer (P -trend = 0.001) but not associated

TABLE 1 Baseline characteristics among cases >55 y of age by ER α and ER β status and controls from the MDC cohort¹

	Controls	Total cases	ER-defined cases					
			ER α +	ER α -	ER β +	ER β -	ER α +	ER α +
<i>n</i>	408	204	178	26	104	100	91	87
Plasma folate, ^{2,3} nmol/L	12.2 ± 8.1	12.9 ± 8.7	13.1 ± 8.5	11.8 ± 7.7	12.2 ± 8.9	13.7 ± 8.5*	12.2 ± 8.7	14.0 ± 9.0*
Time from blood sampling to diagnosis, y		6.3 ± 3.2	6.1 ± 3.2	7.3 ± 3.1	7.5 ± 2.9	4.9 [†] ± 3.0	7.3 ± 3.0	4.9 ± 3.0 [‡]
Food folate, ⁴ μ g/d	227 ± 62	231 ± 74	230 ± 71	236 ± 94	231 ± 77	231 ± 71	230 ± 73	230 ± 70
DFE, ^{4,5} μ g/d	307 ± 447	302 ± 214	306 ± 217	269 ± 195	299 ± 204	305 ± 225	300 ± 196	313 ± 339
Age, y	62.0 ± 4.9	62.0 ± 4.8	62.0 ± 4.9	62.4 ± 4.5	62.4 ± 4.8	61.7 ± 4.9	62.2 ± 4.8	61.8 ± 5.1
Height, m	1.63 ± 6.1	1.63 ± 5.4	1.64 ± 5.6	1.62 ± 4.2	1.64 ± 5.0	1.63 ± 5.9	164.0 ± 5.2*	1.63 ± 6.0
Weight, kg	69.7 ± 12.7	71.2 ± 11.5	71.2 ± 11.5	66.6 ± 10.0	70.6 ± 11.8	70.6 ± 11.0	71.7 ± 11.5	70.7 ± 11.5
Household work, h/wk	19.8 ± 10.7	17.8 ± 9.5*	17.4 ± 9.4*	20.6 ± 10.3	16.8 ± 8.6*	18.9 ± 10.3	16.3 ± 8.0*	18.6 ± 10.5
Menopausal age, y	50.1 ± 4.2	50.2 ± 5.1	50.1 ± 5.1	51.2 ± 5.1	49.4 ± 5.3	51.0 ± 4.7*	49.2 ± 5.4	50.9 ± 4.6
Parity, ⁶ <i>n</i>	2.2 ± 1.2	2.1 ± 0.9	2.1 ± 0.9	2.3 ± 1.0	2.2 ± 1.0	2.1 ± 0.8	2.2 ± 0.9	2.0 ± 0.8
MHT, current, %	18	31*	31*	32	33*	29*	33*	29*
Leisure time physical activity, highest tertile, %	33	34	34	35	30	38	31	37
Socioeconomy, ⁷ high, %	16	23*	24*	19	26*	20	26*	22
Smokers, current or ex, %	48	52	53	42	48	55	50	56

¹ Values are mean ± SD unless noted otherwise. *Different from all controls, $P < 0.05$. [†]Different from ER β +, $P < 0.05$. [‡]Different from ER α + β +, $P < 0.05$, [ANOVA (adjusted for matching variables, age and blood sampling date, when applicable)] or χ^2 test.

² Geometric means were analyzed statistically.

³ Plasma folate was not analyzed for one of the ER α + β - cases.

⁴ Energy-adjusted geometric means were analyzed statistically.

⁵ Dietary folate equivalents.

⁶ Nulliparous women were excluded (25 cases and 43 controls).

⁷ Medium and high collar workers compared with blue-collar workers and low white-collar workers, self-employed participants were excluded in the analysis (13 cases and 29 controls).

with risk of ERβ+ breast cancer (Table 2). The risks for ERβ+ and ERβ- cancer significantly differed (*P*-heterogeneity = 0.003) (Fig. 1). In addition, plasma folate was positively associated with ERα+β- breast cancer (*P*-trend = 0.007), and we observed a similar tendency between plasma folate and ERα-β- breast cancer (*P*-trend = 0.09). The significant association between folate and ERβ- breast cancer remained when the analysis was stratified according to the median time from blood sampling to diagnosis (below median, *P* = 0.01; above median, *P* = 0.04). Plasma folate concentrations were not significantly associated with overall risks of ERα+ or ERα- breast cancers.

Plasma folate concentration and ERβ- breast cancer were positively associated in women both with (*P*-trend = 0.01) and without the *MTHFR* 677T allele (*P*-trend = 0.005) (Table 3). The 677T allele tended to be associated with increased risk of ERβ- breast cancer in all tertiles of plasma folate concentration. We observed the highest risk estimate for ERβ- breast cancer among carriers of the 677T allele with the highest plasma folate concentrations, but there was no statistical interaction between plasma folate concentration and the 677C > T polymorphism. A positive association was also observed between plasma folate and ERα+β- breast cancer independently of 677T status.

TABLE 2 OR and 95% CI of ER-defined breast cancer in tertiles of plasma folate concentrations among cases and controls >55 y of age from the MDC cohort¹

Plasma folate	Tertile			<i>P</i> -trend ²
	1	2	3	
Median concentration	6 nmol/L	10 nmol/L	17 nmol/L	
All women				0.15
Cases/controls, <i>n/n</i>	59/140	62/132	82/132	
OR (95% CI)	1.00	1.11 (0.70–1.74)	1.37 (0.88–2.13)	
ERα+				0.18
Cases/controls, <i>n/n</i>	50/140	56/132	71/132	
OR (95% CI)	1.00	1.19 (0.74–1.93)	1.38 (0.87–2.20)	
ERα-				0.56
Cases/controls, <i>n/n</i>	9/140	6/132	11/132	
OR (95% CI)	1.00	0.45 (0.13–1.49)	1.33 (0.49–3.59)	
ERβ+				0.49
Cases/controls, <i>n/n</i>	39/140	32/132	33/132	
OR (95% CI)	1.00	0.88 (0.50–1.56)	0.82 (0.46–1.44)	
ERβ-				0.001
Cases/controls, <i>n/n</i>	20/140	30/132	49/132	
OR (95% CI)	1.00	1.54 (0.80–2.96)	2.67 (1.44–4.92)	
ERα+β+				0.60
Cases/controls, <i>n/n</i>	32/140	30/132	29/132	
OR (95% CI)	1.00	1.02 (0.55–1.87)	0.85 (0.46–1.56)	
ERα+β-				0.007
Cases/controls, <i>n/n</i>	18/140	26/132	42/132	
OR (95% CI)	1.00	1.48 (0.74–2.94)	2.39 (1.25–4.58)	
ERα-β+				0.26
Cases/controls, <i>n/n</i>	7/140	2/132	4/132	
OR (95% CI)	1.00	0.20 (0.03–1.28)	0.45 (0.11–1.92)	
ERα-β-				0.09
Cases/controls, <i>n/n</i>	2/140	4/132	7/132	
OR (95% CI)	1.00	1.69 (0.23–12.19)	4.20 (0.71–24.66)	

¹ OR were calculated with unconditional logistic regression. Model with adjustment for age, blood sampling date, weight, height, menopausal hormone therapy, age at menopause category, parity, household work category, socioeconomic status, smoking, and alcohol intake category.

² *P*-trend of tertiles plasma folate (treated as a continuous variable).

The increasing risk of ERβ- breast cancer with higher plasma folate concentrations remained after adjustments for total vitamin B-12 intake (*P*-trend = 0.005). There was also an association when excluding women consuming >15 g/d of alcohol (*P*-trend = 0.02) or women consuming folate-containing supplements (*P*-trend = 0.001), as well as in a sensitivity analysis excluding cases diagnosed within 1 y after the blood sampling (*P*-trend = 0.001). The positive association also remained when the analysis was stratified on PR status (*P*-trend = 0.005 and 0.01 for PR+ERβ- breast cancer and PR-ERβ- breast cancer, respectively). In addition, PR status did not modify the association between plasma folate and the other ER-defined breast cancer subtypes (data not shown), and we observed no modification of the overall association between plasma folate and postmenopausal breast cancer (*P*-trend = 0.20 and 0.18 for PR+ breast cancer and PR- breast cancer, respectively). Finally, there were no modifying effects of the *MTHFR* 1298 A > C polymorphism (data not shown).

Discussion

Plasma folate concentrations were positively associated with risk of developing breast tumors with low expression of ERβ. We observed this association in women both with (*P*-trend = 0.01) and without the *MTHFR* 677T allele. There were no significant associations between plasma folate and ERβ+, ERα+, or ERα- breast cancers.

We are not aware of any previous epidemiological study that has taken ERβ status into consideration when examining the association between folate status and breast cancer. In the Women's Health Study, high plasma folate concentrations were associated with an increased risk of ERα+ breast cancer (17), but it cannot be excluded that ERβ status may have confounded those results. We observed a significant association with ERα+β- breast cancer but no association with ERα+β+ breast cancer. On the other hand, it cannot be excluded that high

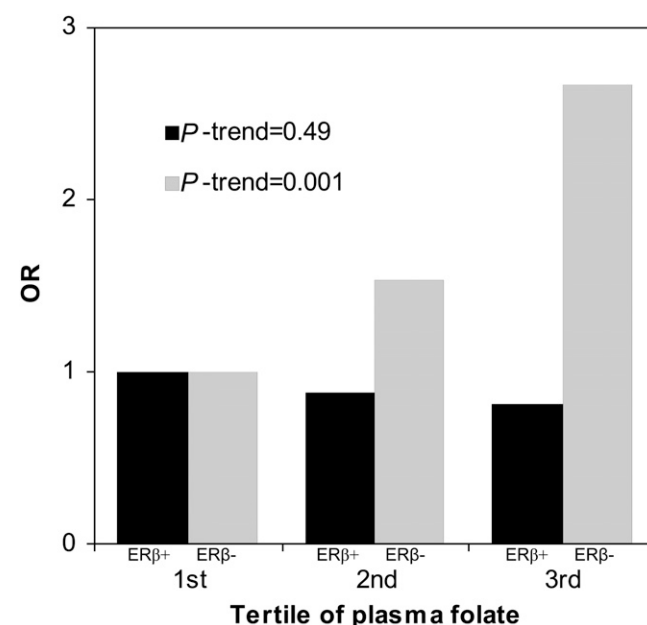


FIGURE 1 OR of ERβ-defined breast cancer in tertiles of plasma folate concentrations among cases (*n*/tertile = 39, 32, 33 for ERβ+, 20, 30, 49 for ERβ-) and controls (*n*/tertile = 140, 132, 132) >55 y from the MDC cohort.

TABLE 3 OR and 95% CI of ER-defined breast cancer according to plasma folate concentrations and *MTHFR* 677 genotypes in cases and controls > 55 y of age from the MDC cohort¹

Plasma folate	Tertile			P-trend ²
	1	2	3	
Median concentration	6 nmol/L	10 nmol/L	17 nmol/L	
ERα+				
677CC				0.39
Cases/controls, <i>n/n</i>	19/61	5/66	34/73	
OR (95% CI)	1.00	1.14 (0.55–2.37)	1.32 (0.66–2.64)	
677CT+TT				0.12
Cases/controls, <i>n/n</i>	31/79	30/65	37/56	
OR (95% CI)	1.16 (0.58–2.34)	1.48 (0.72–3.05)	1.86 (0.92–3.77)	
P-value ³	0.63	0.50	0.14	
ERα–				
677CC				0.38
Cases/controls, <i>n/n</i>	3/61	4/66	7/73	
OR (95% CI)	1.00	0.80 (0.15–4.43)	2.03 (0.45–9.16)	
677CT+TT				0.38
Cases/controls, <i>n/n</i>	6/79	2/65	4/56	
OR (95% CI)	1.27 (0.26–6.09)	0.28 (0.04–2.17)	1.16 (0.21–6.29)	
P-value ³	0.28	0.23	0.42	
ERβ+				
677CC				0.24
Cases/controls, <i>n/n</i>	17/61	17/66	16/73	
OR (95% CI)	1.00	0.80 (0.35–1.79)	0.65 (0.28–1.47)	
677CT+TT				0.91
Cases/controls, <i>n/n</i>	22/79	15/65	17/56	
OR (95% CI)	0.75 (0.34–1.66)	0.72 (0.31–1.69)	0.79 (0.35–1.82)	
P-value ³	0.78	0.89	0.56	
ERβ–				
677CC				0.005
Cases/controls, <i>n/n</i>	5/61	12/66	25/73	
OR (95% CI)	1.00	2.19 (0.70–6.88)	4.23 (1.46–12.24)	
677CT+TT				0.01
Cases/controls, <i>n/n</i>	15/79	17/65	24/56	
OR (95% CI)	2.34 (0.77–7.11)	3.19 (1.04–9.77)	5.78 (1.96–17.07)	
P-value ³	0.04	0.18	0.36	
ERα+β+				
677CC				0.28
Cases/controls, <i>n/n</i>	14/61	15/66	14/73	
OR (95% CI)	1.00	0.86 (0.36–2.05)	0.67 (0.28–1.61)	
677CT+TT				0.95
Cases/controls, <i>n/n</i>	18/79	15/65	15/56	
OR (95% CI)	0.74 (0.32–1.74)	0.88 (0.36–2.16)	0.81 (0.34–1.99)	
P-value ³	0.57	0.95	0.59	
ERα+β–				
677CC				0.03
Cases/controls, <i>n/n</i>	5/61	10/66	20/73	
OR (95% CI)	1.00	1.81 (0.56–5.92)	3.14 (1.05–9.39)	
677CT+TT				0.02
Cases/controls, <i>n/n</i>	13/79	15/65	22/56	
OR (95% CI)	2.16 (0.69–6.73)	3.06 (0.97–9.63)	5.44 (1.81–16.37)	
P-value ³	0.04	0.23	0.10	

¹ OR were calculated with unconditional logistic regression. Model with adjustment for age, blood sampling date, weight, height, menopausal hormone therapy, age at menopause category, parity, household work category, socioeconomic status, smoking, and alcohol intake category.

² P for trend of tertiles plasma folate (treated as a continuous variable).

³ P-value for carriage of the 677T allele in strata of plasma folate tertiles.

expression of ER α is important when occurring in combination with low expression of ER β . Four studies have examined the association between intake of folate and ER α -defined breast

cancer (16,18–20). In accordance with our observations for plasma folate, folate intake was not associated with overall risk of ER α + breast cancer in 3 of the studies (18–20), but results

from the Swedish Mammography Cohort suggest a protective association between folate intake and ER α +PR- tumors (16). The ER α - cases in our study were most likely too few for detection of associations with plasma folate. In the Nurses Health study and in the VITamins And Lifestyle studies, women with high folate intake had lower risk of ER α - breast cancer (18,20). Similar relations were observed among women with an alcohol intake above the median in the Iowa Women's Health Study (19).

ER β expression is frequent in normal breast cells, but decreased expression is present in tumors, and it has been suggested that ER β acts as a tumor suppressor (32). The short time from blood sampling to diagnosis among ER β - cases in this study may support this theory. Folate acts as a methyl donor in biological reactions and may thereby influence DNA methylation (33). Hypermethylation of CpG islands in the promoter region of tumor suppressor genes may lead to gene silencing (34), and DNA methylation has also been associated with silencing of the ER β gene (35). Consequently, it is theoretically possible that folate status outside optimal ranges leads to loss of protective ER β expression in breast cells and thereby promotes development of ER β - tumors. Interestingly, *in vitro* experiments have shown that loss of ER β is a reversible process (36). Knowledge of factors associated with ER β expression may therefore also lead to changes in breast cancer therapy. Today, ER α expression, but not ER β , is clinically established as a predictive marker and used to direct breast cancer treatment (32). A treatment predictive value for ER β expression has been proposed, but the ability to detect the protein varies between different ER β antibodies (36). The ER β antibody used in this study has been validated by comparing the immunohistochemical method with Western blot (37) and can be regarded as valid and specific for ER β (27,38).

The highest plasma folate concentrations (i.e. the third tertile) could have occurred due to consumption of folic acid-containing supplements. Intake of folic acid, instead of naturally occurring folates from foods, may also lead to high concentrations of unmetabolized folic acid. Both increased total folate concentrations and increased concentrations of unmetabolized folic acid have been observed in the US after implementation of mandatory folic acid fortification to prevent neural tube defects (39,40). Whether high levels of circulating folic acid are harmful is not known, but folic acid seems to compete with natural folates in the body (34). Circulating folic acid has also been inversely related to natural killer cell cytotoxicity and may thereby lead to decreased tumor cell destruction (40). Moreover, it has been hypothesized that increased incidences of colorectal cancer in the US and Canada after 1996 and 1998 may be related to folic acid fortification (41), but later studies are not conclusive (42,43). Our results did not change when women reporting consumption of folic acid-containing supplements were excluded. This may indicate that increased risk of ER β - cancer was not caused by folic acid supplements alone. However, some supplement users could have remained in this sensitivity analysis if they did not consume supplements during the specific registration week.

Folate intake in this study population is comparably low (44) and plasma folate concentrations are much lower than, e.g., in the US. The mean plasma folate concentration in participants from the Framingham Offspring Study who did not consume supplements was 23 nmol/L after implementation of folic acid fortification in the US and the prevalence of low folate status (<7 nmol/L) was <2%. However, the plasma folate concentration was only 11 nmol/L before the fortification (45), which is

similar to the levels among nonusers of folic acid-containing supplements in our study. It is possible that the positive associations between plasma folate and ER β - breast cancer could be explained by protective effects of marginal folate deficiency rather than detrimental effects of high plasma folate concentrations. If the highest tertile of plasma folate had been used as the reference, low plasma folate concentration would have been associated with a decreased risk of breast cancer (OR: 0.38; 95% CI: 0.20-0.69; *P*-trend = 0.001). This alternative interpretation is supported by findings from animal studies, because folate deficiency seems to suppress mammary tumor development in rats (46-48). Future studies of other populations may indicate whether elevated folate status among some of the women or high prevalence of low folate status could explain our results.

Strengths of our study are the population-based and prospective design (which minimizes selection bias and reverse causation), the nearly complete follow-up due to the Swedish National Cancer Registry, the extensive information on potential confounding factors, and as the information on ER β status. Plasma folate is regarded as a good marker for total folate status in large epidemiological studies (3), and the energy-adjusted correlation coefficient between folate intake and plasma folate was 0.52 in breast cancer controls from the MDC study (7). In addition, the calculated intra-class correlation coefficient for plasma folate was 0.77 in a subsample of women from the MDC cohort with 3 repeated blood samples [median interval = 34 d (range 19-46 d) between the first and last blood collection] (7). This justifies the classification of women according to plasma folate concentration from the single blood sample used in this study, but folate in plasma almost exclusively appears as 5-methyl THF (49) and may therefore above all reflect the amount of folate available for DNA methylation. Further conversion of methyl groups from 5-methyl THF is, however, also influenced by the vitamin B-12-dependent enzyme methionine synthase. A limitation of the study might be that blood concentrations of vitamin B-12 were not analyzed. In addition, we were not able to examine the interaction between alcohol intake and plasma folate, because the number of women with high consumption of alcohol was too small. Another limitation was the small number of women with ER α - breast tumors, which reduced the power to detect associations between plasma folate concentration and this tumor subtype. Tumor status of both ER α and ER β could be determined for only two-thirds of the cases and the majority of the undefined cases were due to missing data on ER β . This may arouse questions concerning selection of tumors related to other characteristics, such as histology, size, or stage of the tumors. An observation that potentially reduces the risk of differences in tumor characteristics is that the cases with information on ER β status did not differ from those without ER β status regarding major breast cancer risk factors (i.e. age, weight, height, and MHT) (50). Furthermore, differences in tumor characteristics would not explain the different associations between folate and cancers with high or low expression of ER β . Despite adjustments for known risk factors and potential confounders, we cannot completely exclude occurrence of residual confounding. The results also raise questions about the importance of ER β in relation to other lifestyle factors.

This study emphasizes the importance of ER β status of tumors in associations between folate and breast cancer and will possibly affect the design of future studies. Our findings, indicating a positive association between plasma folate concentration and risk of ER β - breast cancer, contribute further understanding of how folate could influence breast cancer

development. If replicated in other populations, the observations may have implications for public health, particularly when considering that one-half of the tumors in this population were defined as ER β -. Together with other studies on folate and disease, the observations may be especially valuable in the ongoing discussions, in many countries, concerning folic acid fortification.

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